ISOLATION OF IAA PRODUCING *ENTEROBACTER* SP. FROM SOIL OF SIMILIPAL BIOSPHERE RESERVE AND ITS EXPLOIT TO SUSTAINABLE AGRICULTURE

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ABSTRACT

The soil microbes produce different plant growth hormones which play a key role in plant growth promotion in natural ecosystem. In the investigation, the IAA producing bacterium was isolated from rhizospheric region of Shorea robusta of Similipal Biosphere Reserve, Odisha, India. The bacterium was identified as Enterobacter sp. by molecular characterization. The production of IAA was optimized and it was found to be maximum production at 6 days of incubation, pH 8 and 370C. Similarly, carbon and nitrogen are key elements for IAA production observed during this study. The highest production of IAA was found at 0.1% of potassium nitrate with 0.5% starch in the broth medium. During In-vivo study, the isolate showed hormonal activity on root formation of Phaseolus mung. So, the study presents the use of bacterium for sustainable agriculture to fulfill our needs in near future.

KEYWORDS: Enterobacter, IAA production, Rhizobacteria & Rhizospheric Bacteria

Original Article

1. INTRODUCTION

Natural soil is exclusive sources of different nutrient for the growth of microbes and plants. The rhizospheric microbes like bacteria, fungi, protozoa and algae promote the plant growth and soil fertility. Therefore, the plant growth promoting rhizobacteria exploits several mechanisms to improve plant growth and health[1,2]. Besides the nitrogen fixation, production of siderophores, solubilization of phosphate and sulphate, synthesis of phytohormones (indole acetic acid, gibberellins and cytokinin) are the major plant growth promotion activities of rhizospheric soil microbes [3,4,5]. The major biosynthesis of plant hormones by rhizospheric bacteria are auxins, gibberellins cytokinin. The bacteria belonging to the genera *Acetobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Gluconoacetobacter*, *Herbaspirillum*, *Klebsiella*, *Ochrobactrum*, *Pantoae*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Stenotrophomonas* etc may act as selective candidates for growth of plant by biosynthesis of different phytohormones. Several *in vivo* study have been demonstrated the potentiality of rhizobacteria for growth of plant in natural condition[6,7,8]. Several researchers reported that auxins produced by soil bacteria to promote the plant growth through cell elongation, cell division, differentiation of phloem and xylem, root initiation, delaying senescence, initiation of flowering, fruits etc [9,10]. The Similipal Biosphere Reserve is reservoir of beneficial microorganisms which need to be exploited in different sectors. Keeping these points in view, the isolates were characterized and optimized for production of IAA.

2. MATERIALS AND METHODS

2.1 Identification

The bacterium isolate S1-7 was found to produce maximum amount of plant growth hormones in comparison to the other isolates by preliminary screening. The isolate was identified by biochemical and molecular tools. The molecular characterization and genotypic identification were performed with partial 16S rRNA sequencing using primer set 518F and 800R. The 16S rRNA of bacterial isolate were sequenced by using forward 518 CCAG CAG CCG CGG TAA TACG and reverse 800 TAC CAG GGT ATC TAA TCC primers. The PCR amplification of genes were carried by Macrogen Inc. South Korea. The sequence analysis of the amplified ribosomal RNA of the isolate (S1-7) with other bacterial 16S rRNA was done using BLAST algorithm.

2.2 Assay for IAA Production

The isolate produced Indole-3-acetic acid on Czapek-dox (150 ml) broth medium containing 0.005M L-tryptophan [11]. The isolate S1-7 was incubated at 37^0 C in dark conditions on a rotary shaker for seven days. After that, the cultures broth was centrifuged at 5000 rpm for 10 minutes. Then, supernatant was taken for quantification and assay of IAA[11]. The 25ml of supernatant was taken and the pH (2.8) was adjusted by 1N HCL. Then, the supernatant was mixed with diethyl ether as solvent and kept in dark (4 hrs). After incubation, the extraction of IAA was carried out by using solvent as diethyle ether (at 4ºC). The extracted solvent was pooled and air dried. The dried extract was stored with methanol solvent. Then, methanol extract (0.5 ml), double distilled water (1.5 ml) and 4 ml of Sapler's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% Perchloric acid) were mixed and kept in dark (1hr) to rise the pink colour. The quantification of IAA was carried out by spectrophotometer study at 535 nm. The amount of IAA present in the supernatant was expressed as mg/25 ml of the medium.

2.3 Optimization of Incubation Period, pH and Temperature

The effect of different environmental factors for production of IAA was optimized by Respond Surface Methodology. The statistical analysis was carried out by using Central Composite Design (CCD) (Stat-Ease, version 8 of Design-Expert) with independent variables as incubation period (X1, day), temperature (X2, ^{0}C) and pH (X3) (Table 1). IAA (Y,mg/25ml) was taken as the dependent variable. The three independent variable as a factor with different coded level (-1.682, -1,0,+1, +1.682) were using in the investigation for determine the range of effect on dependent variable of IAA. A 2^3factorial experimental designed (CCD) with axial points (6nos) and replication (6nos) at the centre point leading to total number of 20 experiment was employed. The statistical significance of the model was studied by Fisher's test and production of variance presented by multiple coefficient of determination, R squared (R^2) [12,2.13].

Table 1: The Coded Level of Independent Variable Determine by RSM for Dependent Variable of IAA

Name of Independent variable	Coded levels				
	-1.682	-1	0	+1	+1.682
Incubation Period (day)	3.6	5	7	9	10.2
Incubation Temperature (ºC)	31.9	34	37	40	42
pH	4.6	6	8	10	11.3

2.4 Optimization of Carbone and Nitrogen Sources

The role of different carbon and nitrogen sources on IAA biosynthesis by the bacterial isolate was studied. The Czapek dox

medium containing L-tryptophan was added with different carbon sources (Fructose, Dextrose, Starch, Sucrose, Sucrose, Xylose, Arbinose, Maltose, Galtactose and Trehalose) at various concentrations (0.5%, 1.0%, 1.5% and 2.0%) The isolate was incubated in the carbon supplement medium and the production of IAA of bacterial isolate was assayed by procedure described earlier. Similarly, the effect of different nitrogen source was studied by supplementing of nitrogen sources (Ammonium Sulphate, Ammonium Nitrate, Sodium Nitrate, Calcium Nitrate, Potassium Nitrate, peptic Digest and Yeast Extracts) at varied concentrations (0.1%, 0.3% and 0.5%). The isolate was incubated in the nitrogen supplemented medium and assayed by procedure as described earlier.

2.5 Statistical Analysis

One way ANOVA was performed using SPSS (version 16) software, to test whether the mean sensory scores of the effect of sources of carbon and nitrogen on IAA production differed significantly from each other or not.

2.6 Analysis of IAA by TLC

The crude extracted IAA was separated by TLC. Methanol extract (10-20µl) was applied on TLC plate (Silica gel Gf 254 and thickness 0.25mm). The mobile phase was prepared by using Hexane-ethyl acetate- water (6:3:1). Spot was detected under UV light at 254 nm, following the procedure of Ehmann [14]. Again the TLC purified compound was analysed by spectrophotometrically.

2.7 Effect of Growth Hormone (IAA) on Plant Growth: A Case Study

Seeds of Mung beans (*Phaseolus mung*) were surface sterilized by 90% alcohol and 2.5% NaOCl. Then, the seeds were washed by sterilized distil water repeatedly. Then similar healthy seeds were cultured into sterile water by overnight. The seeds were taken onto a sterilized petriplate for treatment of growth hormones (8 hrs at 37^0C). The seed treatments were carried out by different condition as applied i.e. bacteria cultured (overnight grown), extracted IAA and standard IAA (1µg/1ml) separately. After incubation, ten seeds were sown into plastic pots at equal depth 1-cm^2 having 200 g autoclaved soil. The pots were moisturized by an equal volume of autoclaved distilled water per day. Root and shoot formation of plant was examined by study length of shoot and number of roots of the plants.

3. RESULTS

3.1 Identification of the Isolated Organism

The isolate S_{1-7} was identified by studying the microscopic and 16S rRNA sequence as *Enterobecter* sp. The rRNA sequence was interpreted and submitted to NCBI data base with accession number **S-KC316118**. BLAST search analysis of the sequence revealed that the species showed closest homology with *Enterobacter* sp. BLAST analysis of the isolate (S1-7) with other bacterial isolates revealed that the isolate clustered within the Enterobacterial group with supported bootstrap of 100%. The evolutionary history of the isolate was studied by using the Maximum Parsimony method. The maximum percentage of parsimonious similarity in tree of the isolate S1-7 with other selected isolates was clustered together is shown. The tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates) [15]. The evolutionary analysis of sequence was conducted by using software MEGA516 and detail Phylogenetic tree is presented in Fig.1.

3.2 Optimization

RSM as a tool will be successfully employed to any process where analyses the effects and interactions   of   different

experimental factors are required. Here, the cultural condition affecting the production of IAA by bacterial sample (S1-7) was studied by using CCD experiments. The findings of CCD experiments for studying the role of independent variable (incubation period, pH and temperature) are presented in Table 2. The production IAA of bacterium was presented in the experiments and the obtained prediction by the model equation (1) are given in Table 2. The ANOVA result of quadratic regression model for Y and Y1 of hormone production IAA is presented in Table 3, where, Y represented the IAA production by isolate. The coefficient (R2) for IAA was found to be 0.96, presenting the statistical model can explain 96 percentage of variability in the response. The value of R^2 is close to 1.0. It is suggesting stronger the model and better it predicts the response[17]. In adequate precision was found to be 15.910 for IAA production. This indicated a good concurrence between the experimental and predicted value for IAA production. The F-value of model was calculated to be 31.59, at probability >F(>0.05) indicating that the model terms are significant for IAA production. The lack of fit F-value was calculated to be 19.45 represent that lack of fit is significant. The response surface of IAA production was presented by plotting on the Z axis against any two independent variable while staying other independent variable at Zero level.

The response surface 3D curves (Fig. 2) described the interactions of independent variables and determine the maximum production of IAA as dependent variable. The maximum orientation of the principal axes of the response surface plot between pH and temperature, incubation period and temperature, temperature and incubation period, designated the mutual interaction between independent variables was found to be significant effect on dependent variable. In the investigation, the three response surface plots for IAA production proved to be significant i.e. incubation period with temperature, temperature with pH and incubation period with pH. A linear increase of production of IAA was found with the increase of incubation period up to 6days (IAA) thereafter, it was declined. A similar result was found in case of pH and temperature for production of IAA. Thus, temperature at 37^0C pH 8.0 were suitable condition for attaining optimum productions for IAA [18,19,,20].

3.3 Model Validation

The model validation was conceded under different variable condition by studying CCD. The experiment values were found to be very near to the predicted values and hence the model was significantly validated and also presenting the accuracy and applicability of RSM of optimization for IAA production (Table 3). The validation of the statistical model and regression equation was performed by taking X_1(6 days), X_2 (37^0C), X_3 (pH 8) in the experiments.

3.4 Effect of Carbon and Nitrogen Sources on IAA Production

The nutrients like carbon and nitrogen regulate microbial production in fermentation process. Therefore, the effects of different carbon and nitrogen sources were studied on biosynthesis of IAA by the isolate. The optimal production of IAA (0.215 mg/25ml) was observed at 0.5% starch and followed by fructose (0.5%, 0.181 mg/25ml), sucrose (0.5%, 0.173mg/ml), (Fig. 3). Similarly, effect of nitrogen source on production of IAA was observed. The highest production of IAA was found to be at 0.1% of potassium nitrate (0.278mg) followed by calcium nitrate(0.233mg), sodium nitrate (0.222), peptic digest (0.161). However, ammonium nitrate and ammonium sulphate inhibited production IAA in the medium (Fig. 4).

3.5 Statistical Modeling of IAA Production in Flask Culture

One way ANOVA was performed to test whether mean sensory scores of the effect of different carbon and nitrogen sources on IAA production differed significantly from each other or not. The effects of different carbon and nitrogen

sources on IAA synthesis by the bacterium were analyzed by One-way ANOVA. The analysis of One way ANOVA showed that all the nine carbon sources differed significantly from each other; i.e starch ($F_{[3,11]}$=464.338, P=0.00); sucrose ($F_{[3,11]}$=237.179, P<0.00); dextrose ($F_{[3,11]}$=50.648;P<0.00); fructose ($F_{[3,11]}$=37.795;P<0.00); maltose ($F_{[3,11]}$=53.457, P<0.00); xylose ($F_{[3,11]}$=149.928; P<0.00); arabinose ($F_{[3,11]}$=146.507, P<0.00); trehalose ($F_{[3,11]}$=52.058, P<0.00); galactose ($F_{[3,11]}$=40.139, P=0.00). Post-hoc analysis revealed that 0.5% starch followed by 1% starch, 0.5% sucrose and 0.5% fructose have significant effect on IAA production (P< 0.05; Tukey's LSD). Further, all other carbon sources were less significantly different from each other. Similarly, the mean scores of seven nitrogen sources which have effect on IAA production were subjected to One-way ANOVA. The Post-hoc analysis revealed that only four nitrogen sources i.e potassium nitrate at 0.1% followed by calcium nitrate (0.1%), sodium nitrate (0.1%) and peptic digest (0.1%) extract have highly significant effect on IAA production (P<0.05; Tukey's LSD). The One-Way ANOVA showed that nitrogen sources had significant effect on IAA production ($F_{[2,8]}$= 121.19; P<0.003) for calcium nitrate; ($F_{[2,8]}$= 49.95; P<0.00) ammonium sulphate; ($F_{[2,8]}$= 1.26; P<0.349) ammonium nitrate; ($F_{[2,8]}$= 4.288; P<0.001) yeast extract; ($F_{[2,8]}$= 0.996; P<0.423) peptic digest; ($F_{[2,8]}$= 89.66; P<0.000) sodium nitrate and ($F_{[2,8]}$= 69.66; P<0.00) potassium nitrate.
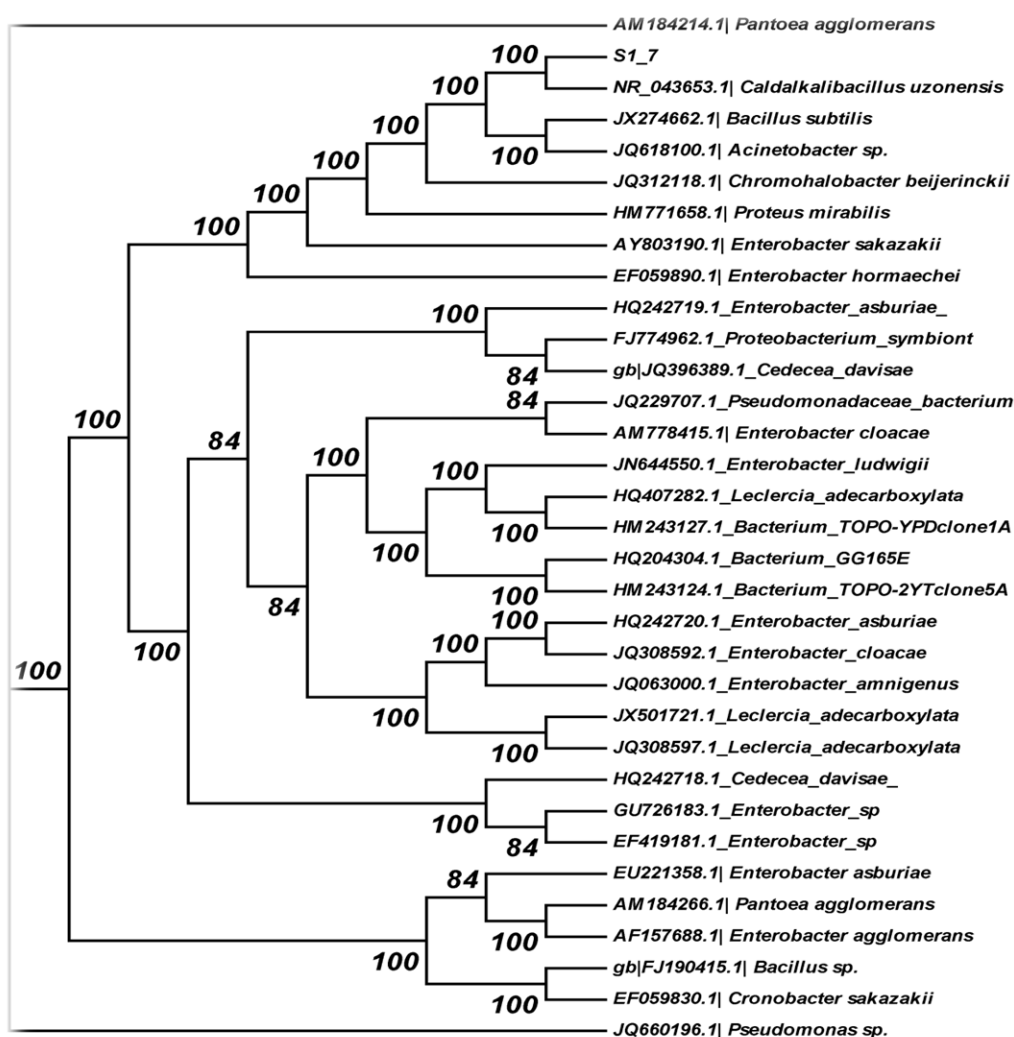

Figure 1: The Evolutionary Relationship of Bacterium (S1-7) along with 32 Closely Related isolates by Maximum Parsimony Study.

Table 2: The Experimental Design by RSM for IAA Production

Standard Order	A: Incubation Period (day)	B: Temperature (^{0}C)	C:pH	IAA Production (mg/25ml)	
				Predicted	Experimental
1	5	34	6	0.031625	0.0281
2	9	34	6	0.057851	0.052
3	5	40	6	0.014193	0.025
4	9	40	6	0.040969	0.041
5	5	34	10	0.029148	0.02
6	9	34	10	0.040924	0.021
7	5	40	10	0.003266	0
8	9	40	10	0.015592	0.01
9	3.6	37	8	0.037344	0.036
10	10.3	37	8	0.069763	0.084
11	7	31.9	8	0.075534	0.094
12	7	42.04	8	0.039573	0.034
13	7	37	4.6	0.005265	0
14	7	37	11.3	-0.01816	0
15	7	37	8	0.128702	0.13
16	7	37	8	0.128702	0.13
17	7	37	8	0.128702	0.12
18	7	37	8	0.128702	0.13
19	7	37	8	0.128702	0.13
20	7	37	8	0.128702	0.13

Table 3: Model validation by ANOVA Study for IAA Production of Isolate in Batch Culture

Sources	Sum of Squares	Degree of Freedom	Mean Square	F -value	P- value
Model	0.048457	9	0.005384	31.59338	< 0.0001
Pure error	8.33E-05	5	1.67E-05		
Total	0.05	19			
R-Squared 0.9660 ; Adj R-Squared 0.9354; Pred R-Squared 0.7514; Adeq Precision 15.910; Lack of fit 19.45					

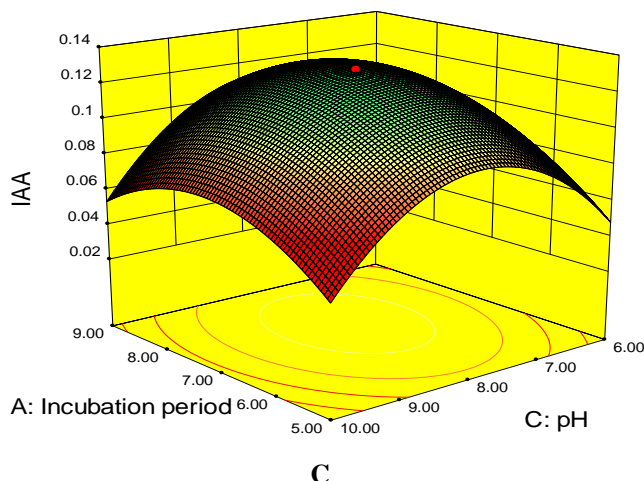A   B
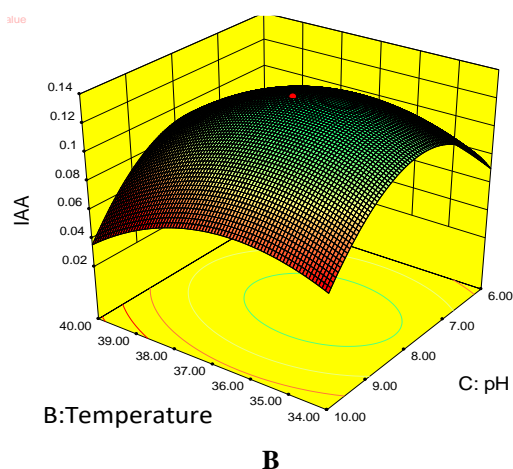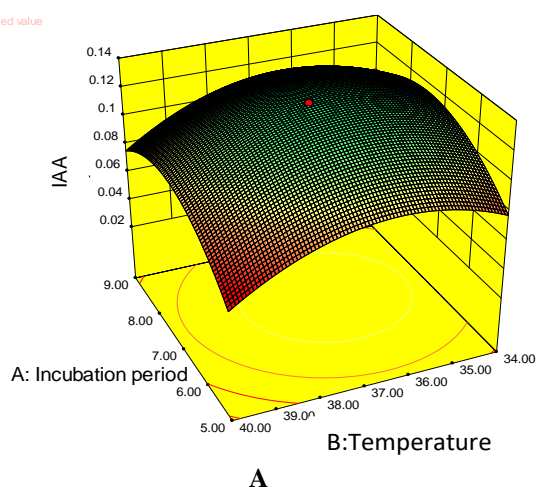
Figure 2: 3D Presentation of Effect of Independent Variable for IAA Production : (A) Incubation Period and Temperature; (B) Temperature and pH; (C) pH and Incubation Period
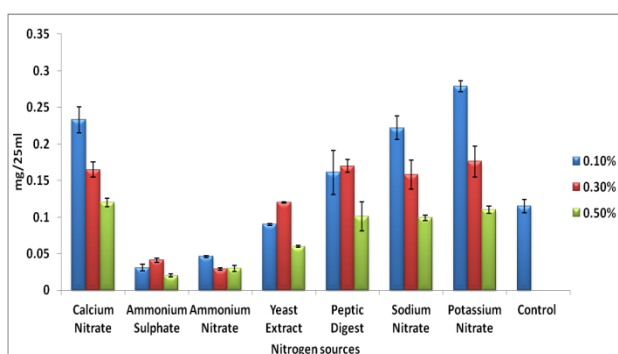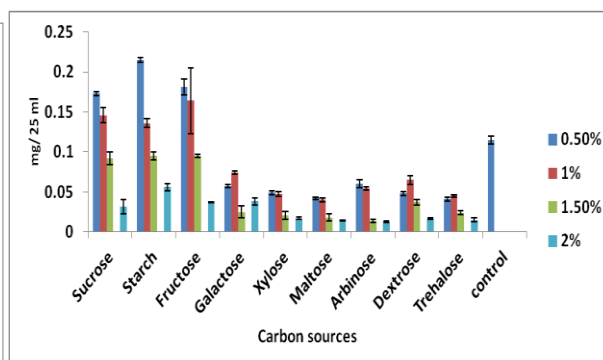

Figure 3: Effect of Carbon on IAA Biosynthesis.

Figure 4: Effect of Nitrogen on IAA Biosynthesis.

3.6 *In Vivo* Study of Effect of IAA on Plant Growth

In this study, an attempt was designed to find out the effect of IAA on plant growth through *in vivo* study. It was found to be significant effect on root formation. The IAA treated seed produced more root in comparison to non treated seed (Table 4, Figure.5). When compared, the result of standard IAA (1µg/ml) and extracted IAA treatment on seed of *Phaseolus mung* it was found that the root formation significantly increases.

**Figure 5: (A) In Vivo Assay of IAA (Produced by Bacterial Isolate S1-7)
(B) Effect of IAA on Root Growth of Phaseolus Mung.**

3.7 TLC Analysis of the Compounds

TLC analysis study of the extracted IAA with standard IAA showed similar R_f values and it was calculated to be 0.89 (fig. 6).
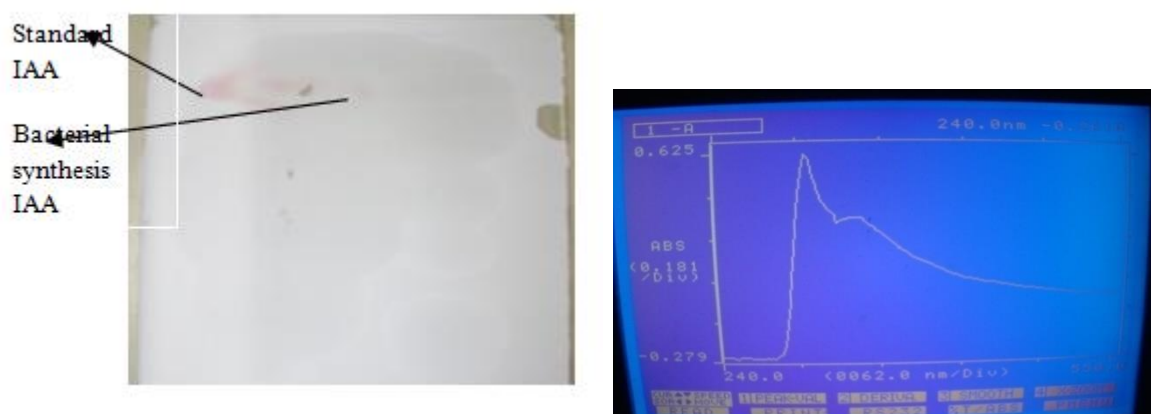

Figure 6: TLC Analysis of IAA Extracted from Isolate (B) Spectrophotometric Scan of Bacterial Synthesis IAA.

Table 4: In vivo Study of IAA on Plant Growth

Condition/Treatments*	Root Numbers	Root Growth (cm)
-Ve Control	4±1	7.0±0.4
Cell Culture	4±1	6.1±0.3
Cell Extracted IAA	7±1.5	5.1±0.2
+Ve Control 1µg/ml	7±1.2	4.2±0.3
*treatment of IAA on seedling		

4. DISCUSSIONS

Production of IAA by soil microorganisms present in the rhizospheric region is an important activity to promote the plant growth.[21,22]. In this investigation, we have elucidated the IAA production by a soil bacterium S1-7 and optimized the production of IAA at different cultural condition. The isolate was identified as *Enterobacter* sp by studying 16SrDNA. In the experiment, we observed production of IAA at 7 days of incubation to be highest. Our findings coincided with Unyaya *et al.* [23] and Hasan [24] reported for *Pseudomonas* sp. that produced optimum IAA during 7 days of incubation in submerged culture i.e. stationary phase of growth. Swain *et al.*,[13] reported that IAA production in *Bacillus subtilis* strains CM4 and CM5 was increased from 2-8 days in tryptophan supplemented culture medium, after that the IAA biosynthesis marginally decreased. The reason behind hypothesized that the bacterium produced maximum IAA in stationary phase might be maximum physiological activity and maximum synthesis of secondary metabolites. When, the effect of pH on biosynthesis of IAA was studied at different pH (4 to 11) it was observed that optimum IAA production by isolate occur at pH 8. However, in a similar study Sahasrabudhe [25] who observed the effect of pH on biosynthesis of IAA by different *Rhizobium* sp. She observed that all rhizobia showed no or little amount of IAA production at pH 5, but maximum production was found at neutral pH. IAA production decreased at higher alkaline and high acidic conditions. It is concluded that highly alkaline and high acidic is not fevour biosynthesis of IAA by bacterium. It has been observed that the optimum production of IAA observed at 37[0]C. This result corroborates to another experiment done by Sachdev *et al.*[26] Where they observed that

Klebsiella strain K8 produced maximum IAA at 37^0C, as reported during our investigation. At very high and low temperature the strain K8 produced very less IAA, as the bacterium was not able to grow at very high and low temperature. We observed that the optimum biosynthesis of IAA by our isolate was at 0.5% starch and 0.1% of potassium nitrate in Czapek dox medium supplemented with L-tryptophan. In contrast to our findings, Sahasrabudhe [25] observed that *Rhizobium loti* and *R. leguminosarum* showed maximum IAA production in presence of glucose followed by lactose and manitol. Basu and Ghosh[27] observed that 1.0% of glucose as a suitable carbon source for synthesis of IAA. It is clear that the effect of carbon sources varied from species to species. We observed more synthesis of IAA in presence of potassium nitrate as a nitrogen source followed by calcium nitrate. However, in another experiment Patil [28] found NH_4Cl (0.1% w/v) as a most suitable source of nitrogen for IAA synthesis by bacterium in broth culture. The statistical method (RCM) has been applied for optimization of IAA biosynthesis in the presence of different independent variable like pH, temperature and incubation period. It is clearly standardized the production of IAA. The TLC analysis of extracted IAA and standards IAA revealed identical Rf values, consistent with previous studies [29]. In- vivo study, plant growth promotion activity of isolate was assayed on *Phaseolus mung* plants and showed that plants treated with extracted IAA and fresh cultured bacterial samples showed the highest root formation. The IAA produced soil bacterium S1-7 was proved as a novel plant growth promoter because it activity was found both *in vivo* and broth culture. So, isolate to be used as PGPR inoculants for plant growth promotion in sustainable agriculture that may enrich soil fertility and enhance crop yields.

5. CONCLUSIONS

A rhizospheric bacterium (S1-7) was isolated from SBR forest and identified as *Enterobacter* sp. through molecular tools. The isolate exhibited the capabilities for biosynthesis of phytohormones like IAA under different environmental and nutritional conditions. The experimental design and findings have been validated significantly. Based on the above observation, it can be concluded that the isolate to be used as potential source of plant growth promoting rhizobacteria in agriculture. However, further studies such as its toxicity, escalation of IAA production is highly essential for exploiting the biotechnological potential of the isolate.

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